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AD_____

AWARD NUMBER DAMD17-97-1-7126

TITLE: Identification of Telomerase Components and Telomerase Regulating Factors in Yeast

PRINCIPAL INVESTIGATOR: Constance I. Nugent, Ph.D.
V. Lundblad

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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# REPORT DOCUMENTATION PAGE

*Form Approved*
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| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TY: | 'ERED |
|---|---|---|---|
| | July 1999 | Annual | (1 Jul 98 - 30 Jun 99) |

**4. TITLE AND SUBTITLE**

Identification of Telomerase Components and Telomerase Regulating Factors in Yeast

**5. FUNDING NUMBERS**

DAMD17-97-1-7126

**6. AUTHOR(S)**

Nugent, Constance, I., Ph.D.
Lundblad, V.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Baylor College of Medicine
Houston, Texas 77030-3498

E*Mail: cnugent@bcm.tmc.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research And Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

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**13. ABSTRACT** *(Maximum 200 words)*

In the yeast *S. cerevisiae*, the *CDC13* gene has been implicated in both the telomerase-mediated pathway for telomere replication and in maintaining chromosome integrity. One model for Cdc13p function at telomeres is that it functions as a single-stranded DNA binding protein and facilitates complete replication, thereby both protecting the chromosome end and potentially regulating access of telomerase to the chromosome terminus. Cdc13p, and its binding partner, Stn1p, may be critical targets for telomere length regulation, as alterations of either gene product can confer telomere shortening or lengthening phenotypes. I found that Cdc13p is phosphorylated in a cell-cycle dependent manner; the functional significance of the phosphorylation is not yet clear. Consistent with a role in DNA replication, Cdc13p appears to be enriched at telomeres during active DNA replication. However, Cdc13p may not function solely at telomeres, as it is also enriched at the centromere of chromosome III during S phase. A panel of mutant *STN1* alleles should prove useful in dissecting its function as well as the nature of its interaction with Cdc13p.

| 14. SUBJECT TERMS | | 15. NUMBER OF PAGES |
|---|---|---|
| Breast Cancer | Telomere, Telomerase, Telomere binding proteins, CDC13, Cancer | 18 |
| | | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Limited |

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Constance Nugent 7/29/99

PI - Signature Date

# Table of contents

# RESEARCH SUMMARY

## INTRODUCTION

Telomeres, the physical ends of linear chromosomes, insulate chromosomes from end-to-end fusion events, thus helping to maintain genetic stability. Telomeres, specialized structures at the ends of chromosomes, help maintain the stability of the genome and are essential for continued cell proliferation. The enzymes that replicate the genome are not capable of fully duplicating the ends of the chromosomes; thus a special mechanism is required to maintain these sequences through replicative cycles (see Nugent and Lundblad for review). In *S. cerevisiae*, telomere length is maintained through the aid of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. In order to replicate completely the chromosome end, telomerase activity is likely to be coordinated with the conventional DNA replication machinery. The goal of my research is to understand the processes involved in duplicating the chromosome ends.

Through genetic and biochemical analysis, the *CDC13* gene has been suggested to encodes a protein critical for telomere replication and length regulation. *In vitro*, Cdc13p binds preferentially to single-stranded G-rich oligomers (Nugent *et al*, Lin and Zakian). Analysis of the *cdc13-1*$^{ts}$ allele revealed that when Cdc13p is absent telomere proximal regions become extensively single-stranded (Garvik *et al*), suggesting either that the replication of the chromosome ends is crippled, unable to properly complete lagging strand synthesis, or that the ends are subject to degradation by nucleases. The *cdc13-2*$^{est}$ allele displays a phenotype similar to that of a telomerase-defective strain, although the level of telomerase activity *in vitro* is normal (Nugent *et al*). The focus of my experiments has been to further understand the function and regulation of Cdc13p and its interacting protein, Stn1p.

## BODY

### *I. CDC13*

### Regulation of Cdc13 protein

*CDC13* is important for both negative and positive regulation of telomere length; thus it might be a key target for signals that modulate telomere length. To investigate whether Cdc13p is a target of regulation, I constructed an integrated, epitope tagged version of *CDC13* [CDC13myc(18x)]. The epitope tag only minimally disrupts *CDC13* function. With this strain, I addressed the following questions.

Is the (steady-state) level of Cdc13p protein cell cycle regulated?
Is Cdc13p modified during the cell cycle?
Is the subcellular localization of Cdc13p cell cycle regulated?

#### *Cdc13p is phosphorylated in a cell cycle dependent manner*

Analysis of Cdc13p in a time course through the cell cycle revealed that a slower electrophoretic mobility band of Cdc13p appears as the cells traverse from G1 into S phase. The intensity of the shifted band accumulates, then disappears as the cells undergo mitosis (Fig. 1). To determine if the slower mobility Cdc13p resulted from phophorylation, immunoprecipitated

Cdc13p was treated with lambda phosphatase. The shifted band collapsed completely after incubation with phosphatase, suggesting Cdc13p is modified by phosphorylation (Fig. 2).

What is the functional significance of the phosphorylation? Phosphorylation of Cdc13p could potentially affect Cdc13p in a myriad of different ways. Among many possibilities, phophorylation could influence the DNA binding capacity of Cdc13p, its association with other factors, protein stability, or subcellular localization. The most straightforward method to determine the significance of the phosphorylation would be to find the residue(s) phosphorylated *in vivo*, and then mutate those sites and analyze the phenotype. I chose to first analyze the ability of Cdc13p to be phosphorylated in a number of mutant kinase strains. None of these kinase-defective strains showed a lack of Cdc13p modification (Table 1). Since other genes involved in maintaining telomere length could potentially indirectly regulate the Cdc13p modification, I also assessed the phosphorylation state of Cdc13p in a number of different mutant backgrounds. Cdc13p phosphorylation was not apparently altered in any mutant tested (Table 1).

**Chromatin association in arrested cells**

Cdc13p isolated from *e. coli* or baculovirus is capable of binding telomeric oligo substrates *in vitro*, suggesting that its modification is not essential for its DNA binding activity. To test whether phosphorylation affects the ability of Cdc13p to associate with telomeric DNA *in vivo*, I tested whether Cdc13p fractionates with chromatin during various stages of the cell cycle. This method fractionates the bulk of the cellular proteins from the bulk of the cellular DNA through a high speed centrifugation step (Donovan *et al*). Since Cdc13p is differentially phosphorylated through the cell cycle, I hoped to determine if its association with DNA *in vivo* correlated with its phosphorylation state. Under the conditions of the assay used, no association with DNA was detected under any of the arrest conditions imposed. Later experiments in which Cdc13p was crosslinked to DNA *in vivo* suggest that the chromatin association of Cdc13p may be sensitive to hydroxyurea, the drug used to synchronize cells in S phase. (see below) Therefore, I will repeat the fractionation experiments to determine if phosphorylated or unphosphorylated Cdc13p associates with chromatin during S phase.

**Sedimentation**

To address the possibility that the phosphorylation state of Cdc13p affects its association with other factors, I tested whether Cdc13p sedimented as part of different sized complexes in G1 (where phosphorylation of Cdc13p is minimal) or pre-anaphase (where phosphorylation of Cdc13p is maximal) arrested cells. The sedimentation profile of Cdc13p in glycerol gradients was similar comparing extracts from G1 arrested or pre-anaphase arrested cells. This data does not rule out the possibility that Cdc13p transiently or loosely associates with different factors at these different cell cycle points. I plan to repeat this experiment to determine if Cdc13p sediments as part of a large complex during S phase.

**Immunofluorescence**

Cdc13p appears to be localized in the nucleus throughout the cell cycle, as determined by indirect immunofluorescence. To the detection limit of this method, there is no correlation between the cell cycle stage (phosphorylation) and Cdc13p localization.

### *In vivo* crosslinking of Cdc13p

#### *Cdc13p is associated with telomeres in a cell cycle dependent manner*

To test the hypothesis that Cdc13p specifically associates with telomeric DNA *in vivo* , the formaldehyde cross-linking method (ChIP) was employed (Aparicio *et al*, Meluh and Koshland). This method allows the assessment of the relative amounts of DNA sequences that immunoprecipitate with the protein of interest after treating cells with formaldehyde, a chemical that reacts with amino and imido groups of proteins and of nucleic acids such that protein-DNA, protein-RNA, and protein-protein crosslinks form between molecules in close proximity (2Å) (Orlando *et al*). Given the data suggesting Cdc13p is regulated in some manner by phosphorylation, crosslinking of Cdc13p to DNA was analyzed in cell cycle synchronized cells. The amount of telomeric DNA ($G_{1\text{-}3}T$) associated with Cdc13p IPs (immunoprecipitations) was enriched during S phase, consistent with a role for Cdc13p in replication of telomeres (Figure 3A). To determine if Cdc13p crosslinks specifically to telomeres, the immunoprecipitated DNA was analyzed for sequences corresponding to regions along the right arm of chromosome V, as well as for the centromeres of chromosomes III and V. Interestingly, the centromere of chromosome III showed a cell cycle dependent enrichment of DNA in the IP similar to the telomeric DNA, whereas none of the other regions probed were greatly enriched in the IPs at any point in the cell cycle (Figure 3B). The significance of the association with CEN3 sequences is not currently understood; Cdc13p does not appear to be a general centromere associated protein since it does not crosslink similarly to CEN5 and *CDC13* mutants do not display phenotypes suggestive of a specific requirement at centromeres.

Experiments are currently in progress to further understand the regulation of the association of Cdc13p with DNA. Preliminary data suggests that the crosslinking of Cdc13 to telomeres and CEN3 is sensitive to hydroxyurea (HU), a drug that inhibits progression through S-phase. Recent data suggests that treatment of *S. cerevisiae* with HU results in the prevention of firing late origins (Santocanale and Diffley, Shirahige *et al.*). Thus, the HU sensitivity of the Cdc13p association with telomeres, regions known to replicate late, is consistent with a role for Cdc13p at telomeres during the process of replication. It is possible that a low level of Cdc13p is constitutively associated with telomeric DNA, and is only enriched at telomeres during replication when more single-stranded substrate is available for binding. Further information concerning how other proteins influence Cdc13p DNA binding *in vivo* will come from analysis of Cdc13p crosslinking in mutant backgrounds.

### Exploring the nature of the defect in the *cdc13-2$^{est}$* allele

Data from two-hybrid experiments suggest that the *cdc13-2$^{est}$* mutation disrupts interaction with *STN1*. To more fully understand the *in vivo* ramifications of the mutation, I epitope tagged mutant cdc13-2$^{est}$ protein and found that neither its protein level nor modification throughout the cell cycle are substantially different from the wild-type protein. Preliminary data, however, suggests that the *in vivo* DNA association of cdc13-2$^{est}$ may deviate from wild-type protein.

## *II. STN1*

### Characterization of *STN1*

*STN1* was originally identified in a screen for low copy suppressors of the temperature sensitivity of *cdc13-1*$^{ts}$ cells (Grandin *et al*). In addition, it was identified in my screen for Cdc13 interacting proteins. The low copy suppression and Cdc13p two-hybrid interaction data suggest that Stn1p and Cdc13p may function as a complex. *STN1* is essential for viability, although its precise function is not known and its sequence reveals no recognizable motifs. High copy expression of *STN1* can lead to telomere shortening whereas mutant *STN1* alleles can display extremely elongated telomeres (Grandin *et al*, Table 2), suggesting that *STN1*, like *CDC13*, is required for proper telomere length regulation. Both genetic and biochemical approaches are being undertaken in order to understand the role of Stn1p in telomere replication. I have generated mutant *STN1* alleles by random PCR mutagenesis as well as by site directed mutagenesis (alanine scanning). These mutant alleles are currently being characterized (Table 2); one goal is to identify an allele that will be useful for identifying additional gene products that interact with *STN1*. To biochemically characterize Stn1p, I have created an epitope tagged *STN1* strain; the protein is detectable in immunoprecipitations and appears to be modified. My focus for the next year is to address the following questions:

- Is *STN1* cell cycle regulated (protein level, modification state, DNA association, subcellular localization) and is such regulation critical for function?
- What is the nature of the interaction between Cdc13p and Stn1p?
  The goal is to determine how interaction of Cdc13p and Stn1p relates to their function in telomere replication and length regulation.
- Can Stn1p be crosslinked to DNA *in vivo*, and if so, does it show a similar or different pattern of DNA association as compared to Cdc13p?
- In addition to Cdc13p, what does Stn1p physically interact with?
  Use the tagged *STN1* strain to look for interaction (by co-IP) with other proteins.
  Identify high copy suppressors of temperature sensitive *stn1* alleles.

### References

Aparicio, O.M., Weinstein, D.M., and S.P. Bell. 1997. Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. *Cell* **91**:59-69.

Donovan, S., Harwood, J., Drury, L.S., and J.F.X. Diffley. 1997. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. . *Proc Natl Acad Sci U S A* **94**:5611-5616.

Garvik, B., M. Carson, L. Hartwell. 1995. Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. *Mol. Cell. Biol.* **15**:6128-6138.

Grandin, N., Reed, S.I., and M. Charbonneau. 1997. Stn1, a new *Saccharomyces cerevisiae* protein, is implicated in telomere size regulation in association with Cdc13. *Genes Dev.* **11**:512-527.

Lin, J.J., and V.A. Zakian. 1996. The Saccharomyces *CDC13* protein is a single-strand TG1-3

telomeric DNA-binding protein *in vitro* that affects telomere behavior *in vivo*. *Proc Natl Acad Sci U S A* **93**:13760-13765.

Meluh, P.B. and D. Koshland. 1997. Budding yeast centromere composition and assembly as revealed by *in vivo* cross-linking. *Genes Dev.* **11**:3401-3412.

Nugent, C.I., Hughes, T.R., Lue, N.F. and V. Lundblad. 1996. Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. *Science* **274**: 249-252.

Nugent, C.I. and V. Lundblad. 1998. The telomerase reverse transcriptase: components and regulation. *Genes Dev* **12**:1073-1085.

Orlando, V., Strutt, H. and R. Paro. 1997. Analysis of chromatin structure by *in vivo* formaldehyde cross-linking. *Methods, Companion Methods Enzymol.* 11:205-214.

Santocanale, C. and J.F. Diffley. 1998. A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature* **395**:615-618.

Shirahige, K., Hori, Y., Shiraishi, K., Yamashita, M., Takahashi, K., Obuse, C., Tsurimoto, T., and H. Yoshikawa. 1998. Regulation of DNA-replication origins during cell-cycle progression. *Nature* **395**:618-621.

**Figure Legends**

**Figure 1.** Cdc13p is modified during S-phase of the cell cycle. A culture of haploid cells containing a single copy of *CDC13* as a fusion with 18 c-myc epitope tags was synchronized in G1 through arrest with alpha factor. (A.) As the culture was released from the alpha-factor block, samples were taken at time points and crude protein extracts were made. For each extract, Cdc13(myc)$_{18}$ was immunoprecipitated from ~ 2 mg of total protein and visualized by western blotting. (B.) FACs analysis was done on cells from each time point to determine their progression through the cell cycle.

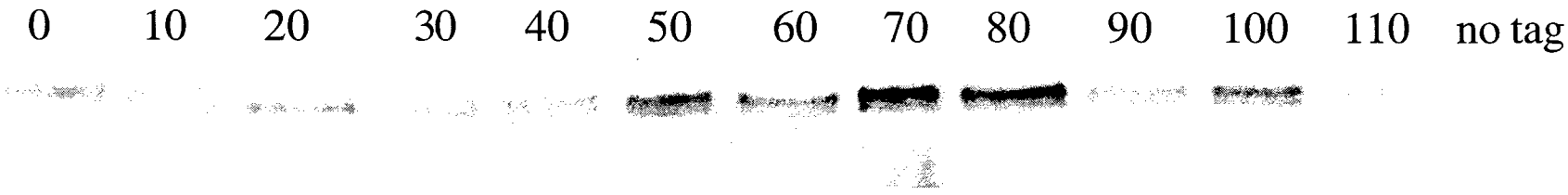
**Figure 2.** Cdc13p is phosphorylated. Cdc13(myc)$_{18}$ immunoprecipitates from nocodazole arrested cells were treated as indicated at 30°C for 45 minutes. The 6% SDS-PAGE gel was blotted and the western probed with 9E10 (anti-myc). (Nocodazole treatment arrests cells pre-anaphase.)

**Figure 3.** (A.) Cdc13p crosslinking to telomeres and *CEN3* is enriched during S phase of the cell cycle. Untagged or Cdc13(myc)$_{18}$ strains were synchronized in G1 through arrest with alpha factor, then released; samples were taken at intervals, treated with formaldehyde for 1 hour, and processed as described in Aparicio *et al.* The 9E10 antibody (anti-myc) was used for the immunoprecipitations. The amount of particular DNA sequences in the immunoprecipitate and input DNA samples was determined by dot blot hybridization. The values plotted represent the fraction of a sequence in the Cdc13(myc)$_{18}$ strain immunoprecipitate versus the fraction of that sequence in the untagged strain immunoprecipitate. (B.) Cdc13p crosslinking is not enriched during S phase at other genomic regions. The immunoprecipitates shown in (A) were probed for sequences at the HMR region of chromosome III, at the RAD24 locus 40 kb distal from the telomere of chromosome VR, and the centromere of chromosome V.

Figure 1. Cdc13p is modified during S-phase.

A. Western blot.

Minutes after release from alpha-factor:




B. FACS analysis.

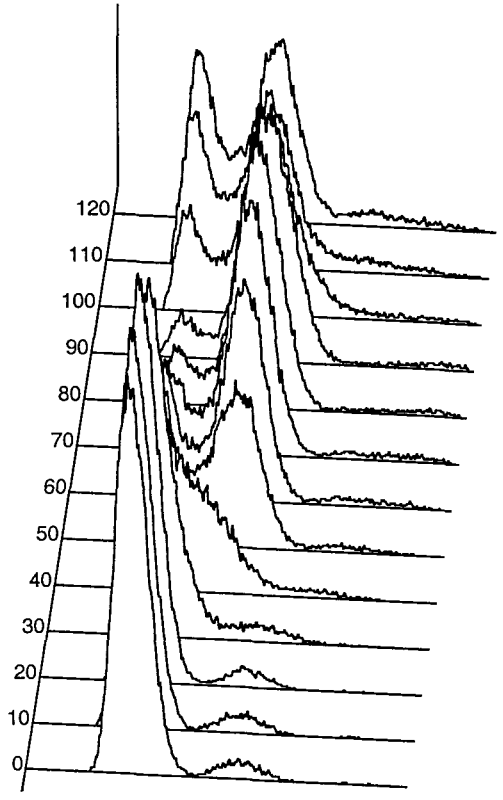


Figure 2.

Cdc13p modification is phosphorylation.

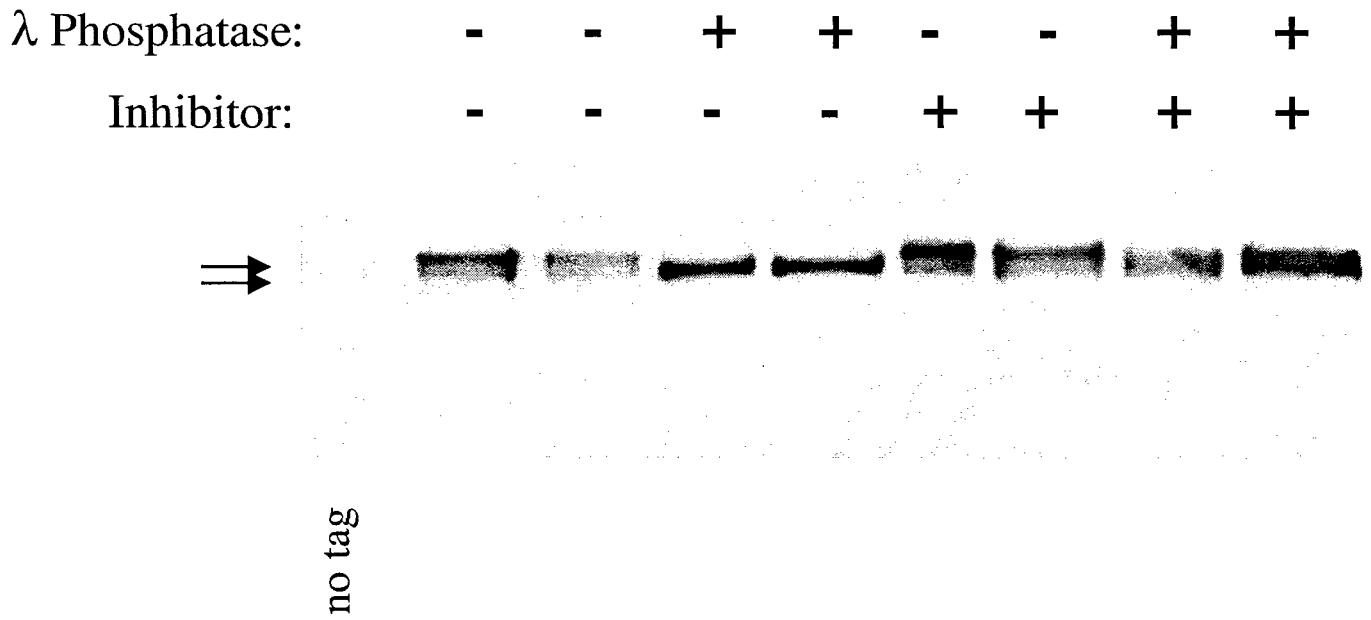


**Table 1.**
**Cdc13 phosphorylation in kinase defective or telomere replication mutant strains.**

Cdc13p remains phosphorylated in the following mutant backgrounds:

| Checkpoint related kinases: | Cell cycle related kinases*: | Telomere defective strains: |
|---|---|---|
| mec1Δ | cdc28-4[a], cdc28-N[b] | tel1Δ |
| mec1-21 | cdc7-1[a] | est1Δ |
| rad53Δ | cdc5-1[b] | est2Δ |
| rad53-21 | | ku80Δ |
| chk1Δ | | mre11Δ |
| dun1Δ | | stn1-13 |

Cdc13p phosphorylation was assessed by determining if the mobility shift pattern on SDS-PAGE was similar to wild-type cells. The phosphorylation state of Cdc13p in the checkpoint related kinase and telomere replication defective strains was assayed in cells arrested with nocodazole. In the strains defective for cell cycle related kinases, Cdc13p was analyzed either in asynchronous cultures incubated at temperatures that should inactivate the kinase (a) or in cultures released from an alpha-factor block at restrictive temperature (b).

**Figure 3. (A)**

**Comparison of Cdc13myc Crosslinking: d(GT/CA) and CEN3 probes**

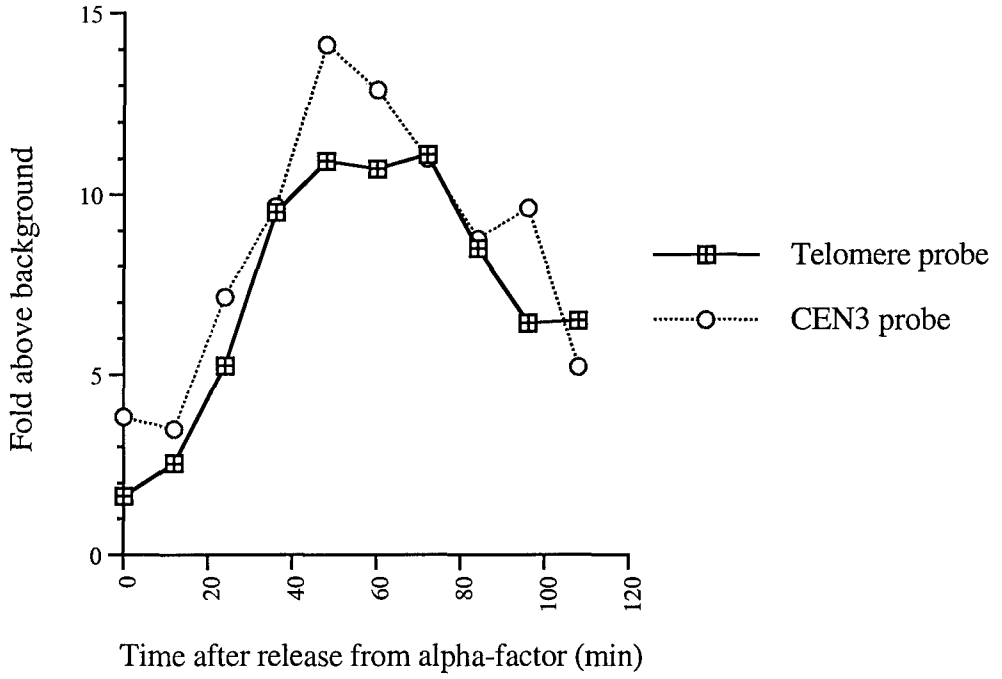
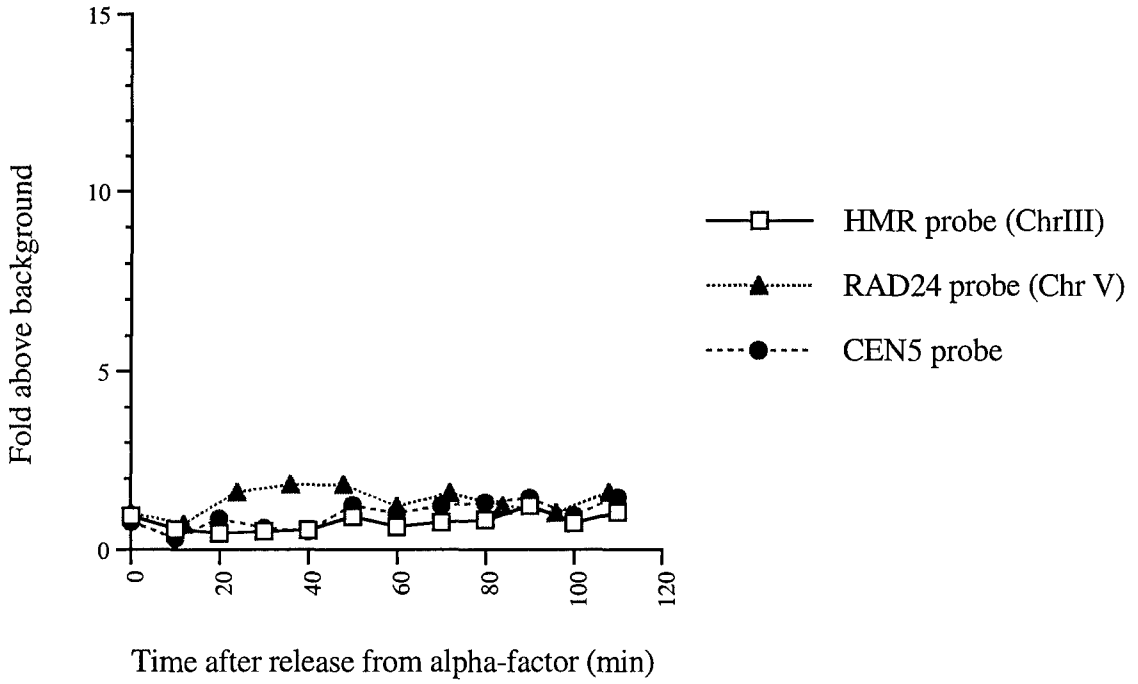



**Figure 3. (B)**

**Comparison of Cdc13myc Crosslinking: additional genomic probes**




Unpublished Data

**Table 2. Characterization of *STN1* mutant alleles.**

| Allele | Growth[a] | ~Telomere length[b] | Two-hybrid interaction with *CDC13* [c] | | Ability of overexpressed mutant to complement null[d] |
|---|---|---|---|---|---|
| | | | *ADE* | *HIS* | |
| **-1** | **sick** | **long** | **+++** | **+++** | **+-** |
| -3 | sick | long | -+ | -+ | + |
| -4 | dead | | - | - | |
| -5 | sick | long | -+ | -+ | + |
| -6 | sick | long | +- | ++ | + |
| -7 | dead | | - | - | |
| -8 | dead | | - | - | |
| -9 | ~dead | long | - | - | |
| -10 | sick | ~wt | - | - | + |
| -11 | ++ | ~wt | +++ | +++ | |
| -12 | ++ | | +++ | +++ | |
| -13 | + | long | +++ | +++ | |
| -14 | ++ | ~wt | +++ | +++ | |
| -15 | ++ | long | +++ | +++ | |
| -16 | + | ~wt | ++ | ++ | + |
| -17 | sick | long | +/++ | +/++ | + |
| -18 | ++ | long | +++ | +++ | |
| -19 | ++ | long | +++ | +++ | |
| -20 | very sick | long | - | - | |
| -21 | ++ | long | +(+) | ++ | |
| **-22** | **++** | **~wt** | **-** | **-** | **++** |
| -23 | + | ~wt | ++ | ++ | |
| -24 | + | long | ++ | ++ | |

a) The growth phenotype indicates the relative health of haploid *stn1Δ* strains containing either the mutant allele or wild-type *STN1* on a plasmid. (++) indicates growth similar to wild-type *STN1*.

b) A more accurate determination of the affect of these alleles on telomere length and temperature sensitivity is being determined. At this point, none of these alleles appear to have a severe temperature sensitive phenotype.

c) The relative strength of interaction of the mutant *STN1* allele with pVL705, the *CDC13* bait, is indicated for two reporters. (+++) denotes growth similar to *STN1* with pVL705, (-) denotes a lack of detectable interaction.

d) The ability of *stn1* plasmids used for the two-hybrid assay to complement growth of *stn1Δ* was tested to confirm that these plasmids do encode some level of protein.

**Key research accomplishments**

- Discovered that Cdc13p is phosphorylated in a cell cycle dependent manner.
- Found that Cdc13p is associated with telomeres in a cell cycle dependent manner.
- Determined that Cdc13p can associate in a cell cycle dependent manner with non-telomeric sequences, such as the centromere of chromosome III.
- Noted that the association of Cdc13p with DNA is sensitive to the drug hydroxyurea, suggesting it functions during late replication.
- Created panel of *STN1* mutant alleles useful for analysis of *STN1* function.

**Reportable outcomes**

Presented talk at interdepartmental seminar at Baylor College of Medicine, April 99

Presented poster at Cold Spring Harbor Telomeres and Telomerase meeting, March 99
Presented poster at FASEB meeting August 98

Abstract of poster presented at FASEB meeting, August 1998:

## THE ROLE OF *CDC13* IN *S. CEREVISIAE* TELOMERE REPLICATION

Constance Nugent and Victoria Lundblad
Baylor College of Medicine, Houston, Texas 77030

Telomere length in many immortal eukaryotic cell populations is maintained at least in part through the action of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. However, telomerase alone is not responsible for maintaining the length and integrity of chromosome ends. In the yeast *S. cerevisiae*, the *CDC13* gene has been implicated in both the telomerase-mediated pathway for telomere replication and in maintaining telomere integrity. One current model for Cdc13p function is that it associates with telomeres as a single-stranded binding protein and facilitates complete replication of the telomeres by both protecting the chromosome end and regulating access of telomerase to the chromosome terminus.

To further explore the role of *CDC13* in telomere replication, we identified two *CDC13*-interacting proteins through a yeast two-hybrid screen. One of these proteins interacts in the two-hybrid assay not only with *CDC13*, but also with *EST2*, the catalytic subunit of telomerase. Although deletion of this novel gene does not lead to perturbations of telomere length, preliminary analysis indicates that there may be some genetic interactions with *CDC13*; further characterization of the function of this gene may reveal some insight into the regulation of telomere replication. The second *CDC13*-interacting protein identified was *STN1*, a gene isolated by M. Charbonneau's lab as a suppressor of the *cdc13-1*$^{ts}$ mutant. In order to understand the role of the Cdc13p/Stn1p complex in telomere replication, experiments are in progress to assess the regulation of these proteins throughout the cell cycle as well as the association of these proteins with chromosome ends *in vivo*. Current data suggests that Cdc13-2$^{est}$p may be unable to interact with Stn1p, suggesting that the *cdc13*$^{est}$ alleles define a domain of interaction with Stn1p that is required for positive regulation of telomere replication. We are also isolating dosage suppressors of temperature-sensitive *stn1* alleles to further elucidate the role of *STN1* at telomeres.

Abstract presented at Cold Spring Harbor Telomeres and Telomerase meeting, March 1999:

## *S. CEREVISIAE CDC13* ENCODES A CELL-CYCLE REGULATED PHOSPHOPROTEIN IMPORTANT FOR TELOMERE REPLICATION

Constance Nugent and Victoria Lundblad
Dept. of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030.

Telomere length in many immortal eukaryotic cells is maintained in part through the action of telomerase, a reverse transcriptase that extends the terminal GT-rich telomeric DNA strand. However, telomerase alone is not responsible for maintaining the length and integrity of chromosome ends. In the yeast *S. cerevisiae*, the *CDC13* gene has been implicated both in the telomerase-mediated pathway for telomere replication and in maintaining telomere integrity. One model for Cdc13p function is that it associates with telomeres as a single-stranded DNA binding protein and facilitates complete replication of the telomeres, thereby both protecting the chromosome end and regulating access of telomerase to the chromosome terminus. To further test this model, I am analyzing the *in vivo* association of Cdc13p with chromatin to determine both the cell-cycle timing and sequence specificity of its binding.

*CDC13* may be a critical target for telomere length regulation, as mutant alleles have been identified that confer telomere shortening or lengthening phenotypes. I have shown that Cdc13p is phosphorylated in a cell-cycle dependent manner, with phosphorylated protein appearing during S phase and accumulating prior to completion of mitosis. Experiments are progress to ascertain both the phosphorylated residue(s) and the functional significance of this phosphorylation.

Using a two-hybrid screen, I identified *STN1* as a *CDC13*-interacting protein, a gene also isolated by M. Charbonneau's lab as a high-copy suppressor of the *cdc13-1*$^{ts}$ mutant. The inability of the senescent *cdc13-2*$^{est}$ allele to interact with *STN1* in the two-hybrid assay suggests that Stn1p may be critical for proper Cdc13p function in telomere replication. I am characterizing additional *stn1* mutant alleles, as well as isolating dosage suppressors of temperature-sensitive *stn1* alleles to further elucidate the role of *STN1* at telomeres.

**DEPARTMENT OF THE ARMY**
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

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